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PLASMID ISOLATION IN LEGIONELLA PNEUMOPHILA AND LEGIONELLA-LIKE--ETC(U)
AUG 80 P MIKESELL, J W EZZELL, G B KNUDSON

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Plasmid Isolation in *Legionella pneumophila* and *Legionella*-like Organisms

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ABSTRACT

Agarose gel electrophoresis was employed to screen nine strains of Legionella-like bacteria and one strain of Legionella pneumophila for the presence of extrachromosomal deoxyribonucleic acid. Cryptic plasmids were found in three of the isolates examined. A purification protocol was devised which consolidates the central tenets of several existing isolation techniques into a single procedure. Plasmid molecular weights were determined to range from 46.6 to 59.8 megadaltons (Mdal).

Approximately one-third of all diagnosed cases of pneumonia in the United States each year cannot be attributed to known viral or bacterial agents (6). Recent reports have established that certain strains of bacteria, designated Legionella-like, are etiological agents of pneumonia in man (2, 11, 13, 17). The Legionella-like isolates examined in this study, WIGA, TEX-KL, TATLOCK, HEBA and five strains of the Pittsburgh Pneumonia Agent (PPA), all possess varying degrees of phenotypic or genotypic relatedness to Legionella pneumophila. The WIGA bacterium isolated in 1959 has been demonstrated to be phenotypically similar but genetically unrelated to L. pneumophila based on deoxyribonucleic acid (DNA) homology studies and may represent a second species of Legionella (D. J. Brenner, A. G. Steigerwalt, G. W. Gorman, R. E. Weaver, J. C. Feeley, L. G. Cordes, H. W. Wilkinson, C. Patton, and B. M. Thomason, Curr. Microbiol., in press). Another WIGA-like bacterium, designated TEX-KL, was obtained from postmortem lung tissue from a patient who died in Texas in early 1979. DNA relatedness studies indicate that TEX-KL may represent a third species of the genus Legionella (13). The 1943 isolate, TATLOCK (9), the HEBA bacterium isolated in 1959 (9) and PPA, isolated in 1979 (19) have identical biochemical, cultural and antigenic characteristics. These strains also are phenotypically similar but genetically unrelated to L. pneumophila (11), indicating that they may represent a fourth species of Legionella (10, 18). The OLDA bacterium, originally isolated in 1947, has now been shown to be a strain of L. pneumophila, serogroup 1 (15). Hereafter we will refer to all isolates as Legionella-like, cognizant of the current taxonomic status of the OLDA isolate.

This investigation was initiated to determine if this group of organisms, like many other medically important bacteria, contain plasmid

elements as part of their genetic complement. We have previously demonstrated the presence of plasmid DNA in both the Atlanta 1 and 2 strains of L. pneumophila (IAI450, Infect. Immun., Sept 80). However, plasmid DNA was not detectable in the Legionella-like bacteria using the cleared lysate technique (1) employed in the previous study, nor by using several alternative plasmid isolation procedures (3, 7, 12). Plasmid DNA was detected in three Legionella-like isolates using an agarose gel electrophoresis technique in which intact cells are lysed directly in the agarose wells prior to electrophoresis. This procedure, unlike many other established methods, does not require preparative lysate treatment. A composite protocol was subsequently developed for plasmid purification. Essential features of this method are elevated temperature incubation of the lysate to enhance cell lysis (8), high salt precipitation (7) and alkaline denaturation of chromosomal DNA (3) and ethanol precipitation of plasmid material (16).

MATERIALS AND METHODS

Bacterial strains. Pseudomonas aeruginosa PU21 obtained from G. A. Jacoby (Massachusetts General Hospital, Boston, Mass.) contains a large 312 Mdal plasmid and a smaller 20 Mdal cryptic plasmid and was used as a control marker with the screening procedure. Escherichia coli V517 was supplied by E. M. Lederberg (Plasmid Reference Center, Stanford, Calif.). This strain was also used as a molecular weight marker for gel electrophoresis and contains eight plasmid species ranging in molecular weight from 1.36 to 35.8 Mdal (14). The Legionella-like isolates OLDA, WIGA, HEBA, TATLOCK and TEX-KL were obtained from the Center for Disease Control, Atlanta, Ga. The five PPA strains (EK, ML, LR, JC, GL) were kindly supplied by A. W. Pasculle (Presbyterian University Hospital, Pittsburgh, Pa.).

Growth conditions. Legionella-like bacteria were cultured on charcoal yeast extract agar (5), yeast extract broth (20) or in a chemically defined media (J. D. Ristroph, personal communication) according to established parameters of growth for L. pneumophila. Control strains were grown on trypticase soy broth or agar.

Agarose gel electrophoresis. Samples were subjected to electrophoresis on a vertical slab gel (BioRad Model 220, Richmond, Calif.) using a BioRad Model 500 power source. Electrophoresis was carried out in 0.8 and 1% agarose (Seakem Marine Colloids, Inc., Portland, Me.) using Tris-borate running buffer and tracking dye as previously described by Meyers et al. (16). Samples were electrophoresed at 2 mA for 60 min followed by 50 mA for 90 to 210 min depending on the degree of band separation desired. Following electrophoresis, gels were stained for 30 min in ethidium bromide (0.5 μg/ml) followed by two 15-min rinses in distilled water. Plasmid bands were visualized using an ultraviolet transilluminator

(Model C-63, Ultraviolet Products, San Gabriel, Calif.) and photographed with Polaroid type 55 or 57 film using combined No. 9 and 23A Wratten gelatin filters (Kodak).

Plasmid screening procedure. Bacterial strains, 1-2 colonies from solid media or cells harvested from 1.0 ml of 40-h yeast extract broth cultures, were screened for the presence of plasmid DNA by the procedure of Eckhardt (4). Lysozyme concentration was increased two-fold and the bacteria-lysozyme mixture in the agarose well was allowed to stand at room temperature for at least 15 min before the addition of the sodium dodecyl sulfate (SDS) mixture. These conditions were required to produce spheroplasts with the Legionella-like bacteria.

Plasmid purification technique. Cells from 100 ml of exponential phase cultures, grown in chemically defined media, were harvested by centrifugation and washed once in 10 ml of 10 mM sodium phosphate buffer, pH 7.0. Washed cells were resuspended in 3.0 ml of 25% sucrose in 50 mM Tris-HCl (pH 8.0), then lysozyme (3.0 mg/ml) was added and the suspension incubated at 37°C in a shaker-incubator. After 15-20 min incubation, 3.0 ml of 250 mM EDTA (pH 8.0) was added and the cells were chilled on ice for 5 min. Cell lysis was achieved by the addition of 1.5 ml of 20% SDS followed immediately by incubation in a 55°C water bath for 5 min with gentle agitation. Freshly prepared 3 N NaOH was added dropwise until the pH was 12.1 to 12.4. The pH was immediately reduced to 8.5 to 9.0 with 2 M Tris-HCl (pH 7.0). Denatured chromosomal DNA and cellular debris were precipitated by the addition of 1.5 ml of 20% SDS and 3.0 ml of 5 M NaCl followed by overnight storage at 4°C. The following day the lysate was centrifuged for 30 min at 17,000 x g at 4°C. The precipitate was discarded and RNase (2 mg/ml in distilled

water, heated to 100°C for 5 min) was added to the supernatant to a final concentration of 100 μg/ml and incubated for 30 min at 37°C. Plasmid DNA was precipitated by the addition of 1/20 vol of 3 M sodium acetate and 2 vol of cold 95% ethanol and stored at -20°C for at least 4 h. Plasmid DNA was concentrated by centrifugation for 30 min at 17,000 x g and the resultant pellet resuspended in 100 to 200 μl of Tris-borate buffer. This purified preparation was suitable for electrophoresis.

Molecular weight estimates. Escherichia coli V517 was employed as an internal molecular weight standard for agarose gel electrophoresis. Least squares regression analysis as described by Hansen et al. (8) was used to estimate unknown plasmid molecular weights based on relative migration rates of the plasmid bands with respect to the control markers.

RESULTS AND DISCUSSION

Plasmid detection by electrophoresis. The use of the screening technique described in Materials and Methods resulted in plasmid band migration patterns similar to the purified DNA preparations (data not shown). As expected, there was an increase in contaminating debris when intact cells were lysed directly in the agarose well. The resolution of plasmid DNA was markedly increased with cells harvested from broth cultures compared to cells harvested from solid media. This may either reflect interference of cell lysis by components in charcoal yeast extract agar or cells grown on solid media may be less susceptible to lysis. Cell concentration and improper mixing of cells in the well also affected the resolution of DNA bands. This procedure was used only as a screening techniques was not used for definitive characterization of the plasmid species.

DNA purification. The migration patterns of purified plasmid DNA from the OLDA, WIGA and TEX-KL isolates are shown in Fig. 1. Molecular weight estimates were determined from the relative migration rates of plasmid bands in agarose gels (Fig. 2). The OLDA strain of L. pneumophila contained a single convalently closed circular plasmid species, pLP3, (Fig. 1A) with an estimated molecular weight of 59.8 Mdal. This is the largest of the five plasmid isolates. The WIGA bacterium contained two plasmid species pLB1 (54.3 Mdal) and pLB2 (47.6 Mdal) (Fig. 1B), the smaller of the two having a double band appearance. This third intermediate band is believed to be a catenated form of the smaller of the two plasmids (pLB2) in the WIGA isolate and not an open circular form of pLB2 or a third distinct plasmid species. Further evaluation of this observation using electron microscopy is in progress. The TEX-KL

organism also has two plasmid species pLK1 and pLK2 with molecular weights of 58.6 and 46.6 Mdal, respectively, (Fig. 1C). The alteration of selected electrophoretic parameters shows that all plasmid isolates are unique entities. Plasmid DNA was not detected in the HEBA, TATLOCK or five PPA isolates by either the screening procedure or the composite purification technique. The failure to isolate extrachromosomal DNA from these organisms may be due to shortcomings in the techniques; alternate methodologies may eventually establish the presence of plasmid DNA in these organisms. The small 20 Mdal cryptic plasmid of the control P. aeruginosa strain was not observed using either procedure. It is possible that this culture has been cured of this smaller plasmid, since extrachromosomal DNA from E. coli in the 10 to 20 Mdal range has been successfully resolved by our techniques (data not shown).

The recovery of plasmid DNA from cells grown in complex undefined media is very low compared to recovery of plasmid material from cultures grown in chemically defined media. This observation may reflect the same lysis interference phenomena noted with the in-well screening procedure. Routinely, we now attempt to culture unknown and control bacterial strains in chemically defined media. Attempts to enhance plasmid recovery using phenol extraction or heat-pulse (8) rather than heat elevation also resulted in lower yields of DNA. Although these plasmid species do not have high molecular weights, they appear to be highly susceptible to shear forces which may explain our initial failures in attempting to isolate extrachromosomal DNA by conventional procedures.

The isolation of extrachromosomal DNA from members of Legionella is not surprising, in consideration of the ubiquitous nature of plasmid elements. These results indicate that members of this genus, like other human pathogenic microorganisms, are able to stably maintain plasmid

DNA as part of their total genetic complement. The plasmid content of each proposed species is unique and may possibly be used in the future as one of several considerations for classification within the genus Legionella.

The ability of these microorganisms to exchange genetic information with other bacteria has, to our knowledge, not been reported. However in view of the narrow spectrum of antibiotics effective in the treatment of diseases caused by L. pneumophila and Legionella-like organisms, the acquisition of drug resistance or virulence factors by these bacteria could have serious clinical ramifications. Investigations are in progress to determine if the plasmid isolates contribute to the virulence of these novel pathogenic microorganisms.

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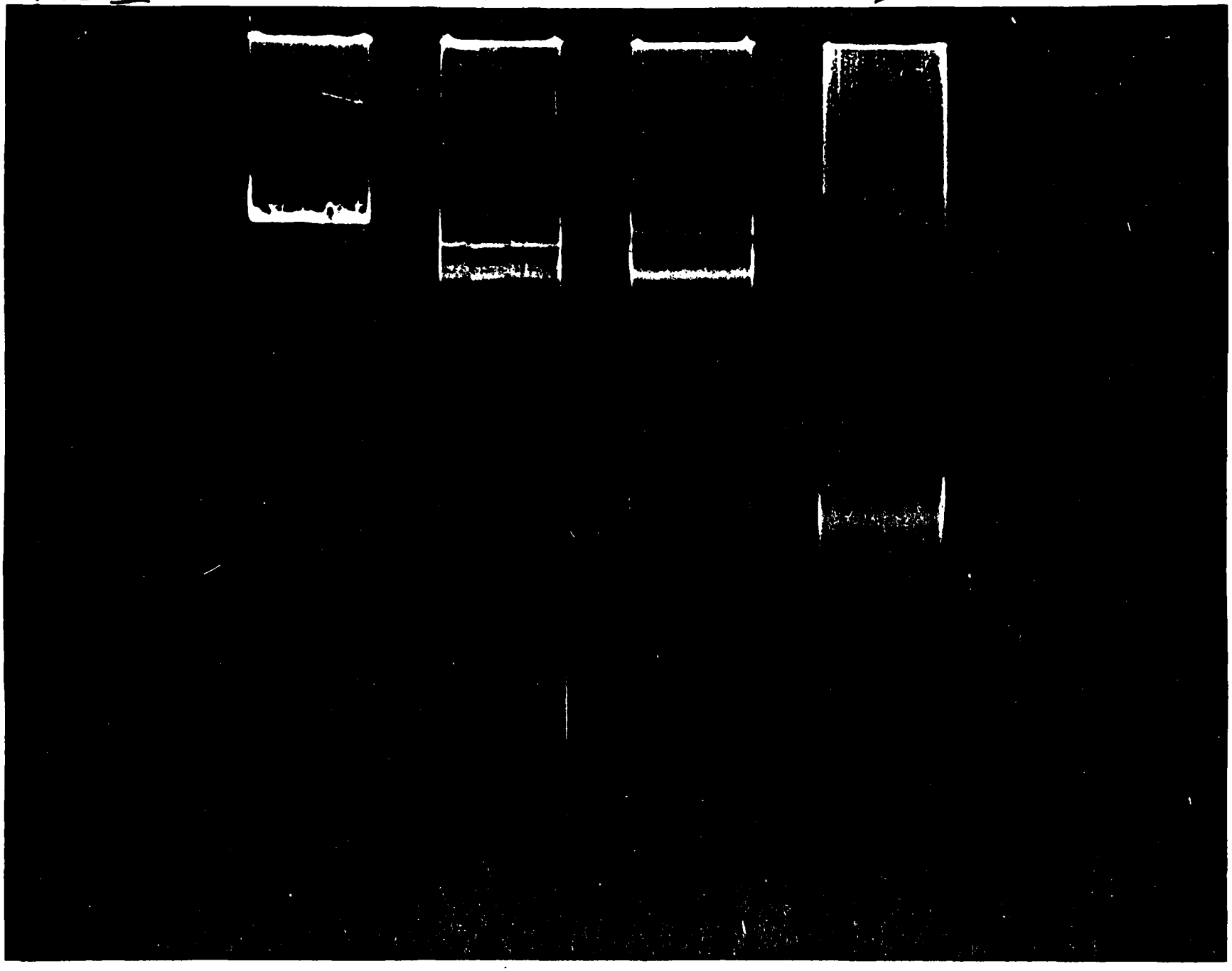
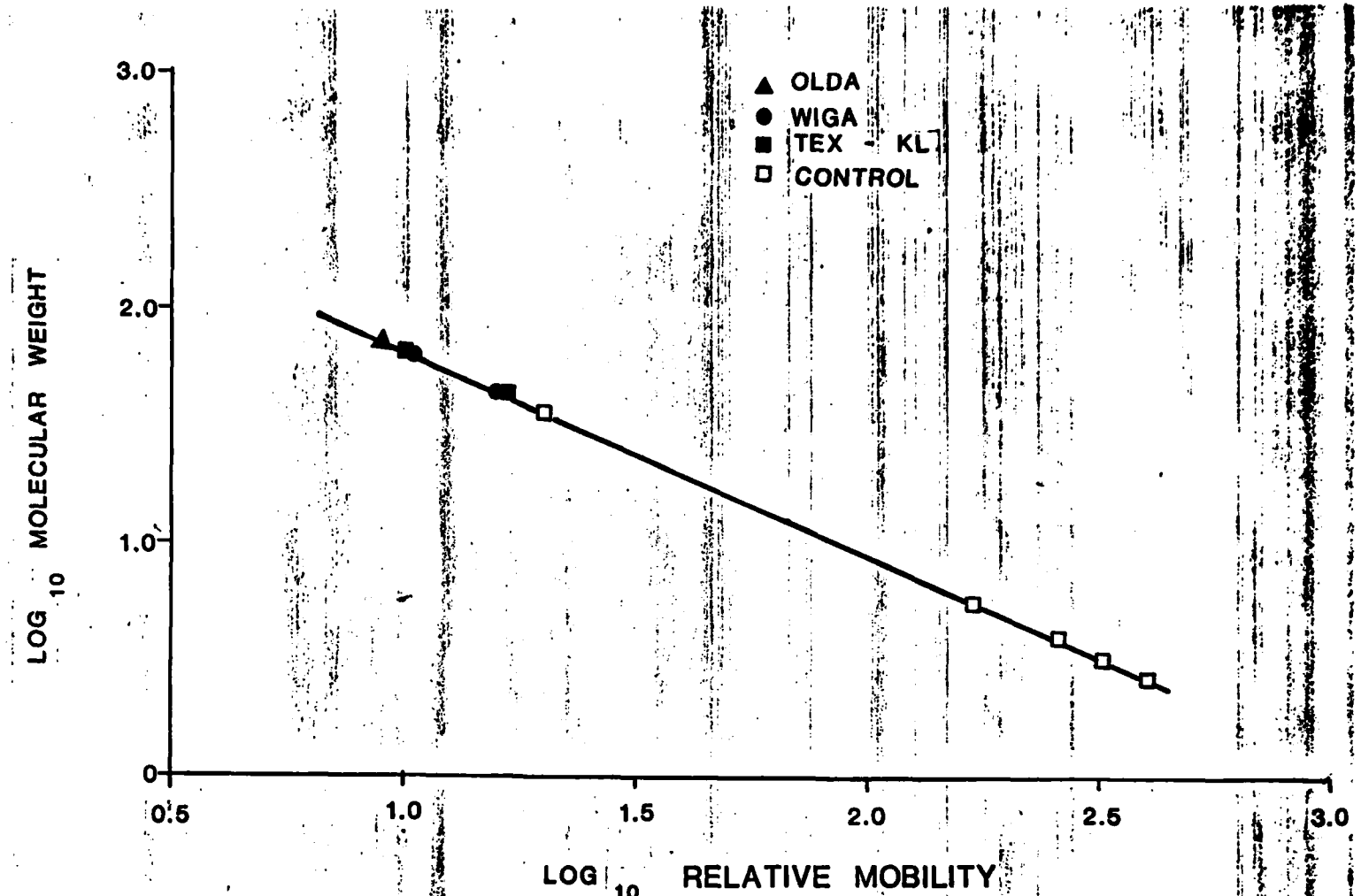
FIGURE LEGENDS

FIG 1. Gel electrophoresis of purified plasmids from Legionella-like bacteria. 30 µl of purified DNA was mixed with 40 µl of tracking dye. 30 µl of DNA-dye mixture was applied to agarose well. DNA samples were subjected to electrophoresis in 1% agarose at 2 mA for 60 min followed by 50 mA for 210 min. (A) OLDA strain of L. pneumophila, (B) WIGA isolate, (C) TEX-KL strain, (D) P. aeruginosa, PU21 control.

FIG 2. Least-squares regression analysis of plasmid DNA. Plasmid DNA from E. coli V517 was purified by cesium chloride-ethidium bromide buovant density centrifugation. Plasmid molecular weights of marker strain are 35.8, 4.8, 3.7, 3.4 and 2.6 Mdal. The three smaller plasmid species of the marker strain (2, 1.8 and 1.4 Mdal) were not retained on the gel under the stated electrophoretic conditions.

FIG 1




OLDA
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TEX - KL
CONTROL
LOG 10 MOLECULAR WEIGHT
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LOG 10 RELATIVE MOBILITY

Fig. 2